

Prognostic value of chromosomal imbalances, gene mutations, and BAP1 expression in Uveal Melanoma.

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ABSTRACT

Uveal melanoma (UM) exhibits recurring chromosomal abnormalities and gene driver mutations, which are related to tumor evolution/progression. Almost half of the patients with UM develop distant metastases, predominantly to the liver, and so far there are no effective adjuvant therapies. An accurate UM genetic profile could assess the individual patient's metastatic risk, and provide the basis to determine an individualized targeted therapeutic strategy for each UM patients.

To investigate the presence of specific chromosomal and gene alterations, BAP1 protein expression, and their relationship with distant progression free survival (DPFS), we analyzed tumor samples from 63 UM patients (40 men and 23 women, with a median age of 64 years), who underwent eye enucleation by a single cancer ophthalmologist from December 2005 to June 2016. The presence of losses/gains in chromosomes 1p, 3, 6p and 8q was determined by MLPA; GNAQ, GNA11, BAP1, EIF1AX and SF3B1 genes were sequenced, and BAP1 protein expression was detected by immunohistochemistry (IHC).

Multivariate analysis showed that the presence of monosomy 3, 8q gain, and loss of BAP1 protein were significantly associated to DPFS, while BAP1 gene mutation was not, mainly due to the presence of metastatic UM cases with negative BAP1 IHC and no BAP1 mutations. Other mechanisms than mutation might be responsible for the loss of BAP1 protein expression, which, together with monosomy 3, represent the strongest predictors of metastases, and may have important implications for implementation of patient surveillance, properly designed clinical trials enrollment, and adjuvant therapy.

INTRODUCTION

Uveal melanoma (UM) is a rare malignancy arising from uveal melanocytes. Current diagnosis of UM is based on both the clinical experience of the specialist and diagnostic techniques such as indirect ophthalmoscopy, ultrasonography scans, fundus fluorescein angiography, and transillumination¹. Despite the wide range of therapeutic options, including local radiotherapy, surgical resection and phototherapy, almost half of the patients with UM develop distant metastases, predominantly to the liver². The poor outcome of UM patients with metastatic disease derives from the absence of proven effective adjuvant therapies^{3,4}. UM prognosis is based on clinicopathologic factors, and, most importantly, on molecular and genetic markers⁵⁻¹⁰.

UM typically exhibits recurring chromosomal abnormalities, which are related to tumor progression¹¹. Recently, Yavuziyigitoglu et al¹² hypothesized that UM with specific mutations are characterized by different mechanisms causing different types of chromosomal abnormalities. Chromosome alterations correlated with reduced UM patient survival are chromosome 3 loss, chromosome 8q gains and loss of chromosome 1p (reviewed in 3). UM also shows a pattern of recurrent mutations. Mutations in GNAQ and GNA11 are early events that promote cell proliferation. Mutations in BAP1, SF3B1, and EIF1AX represent later events that are largely mutually exclusive. BAP1 mutations are associated with rapid metastatic progression and EIF1AX mutations with prolonged metastatic-free survival¹³⁻¹⁵. SF3B1 mutations have an intermediate risk and are associated with late metastasis¹⁶.

Predictive information on the clinical outcome of UM patients could provide, in a hopefully near future, the basis to determine an individualized, targeted therapeutic strategy for each UM patient. To reach this goal, it is important to have an accurate prediction system to assess the individual patient's metastatic risk.

In this study, we evaluated clinical, pathologic, and genetic features of a UM series, and analyzed their associations with metastatic progression.

MATERIALS AND METHODS

Patients

Tissue samples were obtained from 63 primary UM after enucleation surgery at E.O. Galliera Hospital, Genoa, Italy, between December 2005 and June 2016. Written informed consent was obtained from all patients. Follow-up data were available for 61 patients, as shown in Table 1.

Tissue sampling and histopathological analysis

Tumor sampling was performed directly after enucleation by fine needle aspiration biopsy or by transscleral biopsy. For histopathologic examination, the eyes were formalin fixed and paraffin embedded (FFPE), and 2 µm-thick sections were stained with the conventional hematoxylin and eosin stain. Two co-authors (RB and MR) confirmed the diagnosis of UM. Cell type was assigned according to the modified Callendar classification system¹⁷.

DNA extraction and Sanger sequencing

DNA was extracted from tumor material conserved in RNA/aterTM (Ambion, Monza, Italy) or from archival FFPE blocks using QIAamp DNA Mini kit and QIAamp DNA FFPE MINI kit (Qiagen, Hilden, Germany), respectively, according to the manufacturer's instructions. DNA concentration and quality were checked in the Nanodrop-ND-1000 spectrophotometer and integrity of DNA by agarose gel electrophoresis. PCRs and sequencing data analysis were carried out as previously published¹⁸. All PCR primers are reported in in the Supplementary Table1.

Multiplex ligation-dependent probe amplification (MLPA)

Multiplex ligation-dependent probe amplification (MLPA) was performed according to the manufacturer's instructions (MRC Holland, Amsterdam, The Netherlands). In short, 100 ng of genomic DNA diluted in 5 µl TE were denatured at 98°C for 5 min, cooled to 25°C and 3 µl of a 1:1 mixture of MLPA buffer and SALSA P027-C1 Uveal Melanoma probe-mix was added. After hybridization for 16 h at 60°C, 32 µl ligation mix were added, and the reaction was incubated for 15 min at 54°C followed by 5 min at 98°C. Subsequently, 10 µl of the SALSA PCR-mix were added to 40 µl of ligation product and this was amplified by PCR in 35 cycles (30 s, 95°C; 30 s 60°C; 60 s 72°C). PCR products were quantified using the ABI 3130XL (Applied Biosystems-Life Technologies Corporation, Milan, Italy) and coffalyser.net software (MRC-Holland). Data were analyzed as already published¹⁷.

Microsatellite analysis (MSA)

Twelve polymorphic microsatellite repeats on chromosome 3 were chosen to perform MSA (Supplementary Table 2). PCRs were carried out using 25 ng of genomic DNA in a 25 µl reaction mix including 10x Platinum® PCR Supermix, 1.5mM MgCl₂, 200µM dNTPs, 0.4µM each primer pair and 2U Taq Platinum (Invitrogen-Life Technologies Corporation, Italy). PCR cycles: 95°C for 12 min, 10 x (15s, 94°C; 15s 56°C; 30s 72°C), 20 x (15s 89°C; 15s 56°C; 30s 72°C); 30m 72°C. PCR products were quantified using the ABI 3130XL sequencer (Applied Biosystems-Life Technologies Corporation, Milan, Italy) and analyzed by Genemapper™ software (Applied Biosystems, Foster City, CA, USA). A comparison of the peak area of DNA from UM biopsy specimens and normal DNA from matched blood sample allows the determination of allele ratio in UM.

Sodium bisulfite modification and Pyrosequencing Assay

Genomic DNA was chemically modified with the Epitect Bisulfite kit (Qiagen, Milan, Italy). The PCR and sequencing primers for BAP1 were designed with the Pyrosequencing Assay Design Software (Biotage, Uppsala, SW) to recognize some CpG sites in the CpG island in the TSS defined as Area 1¹⁹. The PCR primers sequences were: BAP1 Fw: 5'-Bio-GAGGGAGGGTTTGGATATG-3'; BAP1 Rev: 5'-ATCCCCTCCTCACCTAAA-3'. The resulting amplicons were pyrosequenced (SPQ 96MA instrument, Qiagen) utilizing the Pyro Gold reagent kit SPQ 96MA according to the manufacturer instructions (BAP1 Sequencing primer: 5'-CCCCTCCTCACCTAAA-3'). Sequence analysis was conducted with the Pyro Q-CpG software (version 1.0.9).

BAP1 Immunohistochemistry (IHC)

IHC was performed on 2 µm sections with an automated IHC staining system (Ventana BenchMark ULTRA, Ventana Medical Systems, Italy). After deparaffinization and heat-induced antigen retrieval, the sections were incubated with a mouse monoclonal antibody raised against amino acids 430-729 of human BAP1 (C-4 clone sc-28383, 1:50 dilution, Santa Cruz Biotechnology, Inc. USA), followed by incubation with haematoxylin II. Endothelial cells constituted the internal positive control for IHC procedure. Two independent observers (RB and MR) scored the slides with no prior knowledge of the UM chromosome 3 status.

Statistical Analysis

Fisher's exact test was used to detect associations between the prevalence of metastasis and main discrete dichotomous factors. Univariate survival analysis of time to metastasis was performed using the Kaplan–Meier method (log-rank test), considering the date of enucleation as starting point and the date of MT diagnosis as endpoint. Data were censored on the date of last follow-up. Cox Proportional Hazard modelling was employed to estimate hazard ratios (HRs), adjusting for age, tumor stage (T), and sex. Statistical analysis was performed using STATA statistical package (version 14, Stata Corp, College Station, Texas, USA).

RESULTS

Clinical-pathological findings

The study cohort comprised 63 UM patients, consisting of 40 men (63%) and 23 women (37%), with a median age at the time of eye enucleation of 64 years (range 28-89 years) and a median follow-up time of 3 years (interquartile range, IQR: 2-6 years). Thirty-five out of 63 (56 %) patients had developed uveal melanoma metastases to the liver (mean time to metastasis 25.7 months; range 0–97 months). All the data relating to the patients, the pathological T stages, the UM histological cell type, and follow-up are summarized in Table 1.

MLPA and MSA

The distribution of gains in chromosomes 8q and 6p, chromosomes 3 monosomy/isodisomy, and losses in 1p is shown in the first 4 rows of Figure 1. MLPA was successful in 60/63 (95%) UM samples. Forty out of 60 (67%) analyzed samples harboured monosomy 3, 5/60 (8%) a partial monosomy 3 (Figure 1 and Supplementary Figure 1), 13/60 (22%) the loss of the short arm of chromosome 1 (1p-), 17/60 (28%) gains in 6p, and 41/60 (70%) gains in 8q. In 57 UM samples, the monosomic/disomic status of chromosome 3 was also confirmed by chromosome 3 MSA (MSA failed in UM01, UM06, UM10, UM40, UM49, and UM54). In 4 UM samples (UM2, UM20, UM33, and UM34) with a normal chromosome 3 dosage by MLPA, loss of heterozygosity (LOH) of all informative MSA markers indicated isodisomy of chromosome 3. In UM17, in addition to 1p loss, 6p gain and 8q gain, MLPA showed the loss of control probes in 2p12.3, 4q13.2, and 13q14.3 (data not shown).

Mutation analysis

GNAQ mutations were detected in 20/63 (32%), and GNA11 mutations in 25/63 (40%) UM samples (Figure 1 and Table 2). In GNAQ, all the mutations were missense mutations at the two known hotspots Q209 (13/20 (65%) c.626A>C p.Q209P, and 6/20 (30%) c.626A>T p.Q209L), and R183 (1/20 (5%) c.548G>A p.R183Q). In GNA11, all the mutations were missense mutations at the two known hotspots Q209 (23/25 (92%) c.626A>T p.Q209L), and R183 (2/25 (8%) c.547C>T p.R183C).

BAP1 coding regions and splice sites sequencing was successful in 60/63 (95%) UM samples, due to poor DNA quality/quantity of 3 samples (UM9, UM14, and UM49). BAP1 mutations were identified in 31/60 (52%) UM samples: 28 cases with monosomy 3 and 3 with isodisomy 3 (Figure 1, and Table 2). In 6 UM samples with monosomy 3 (UM16, UM28, UM31, UM36, UM43, and UM48), Sanger sequencing showed a BAP1 heterozygous mutation (Table 2). Thirteen out of 31 (42%) UM had in-frame mutations, consisting in 10 missense mutations and 3 in-frame deletions. To predict the possible impact of the amino acid substitutions due to the different missense mutation on the structure and function of BAP1 protein, Polyphen-2²⁰ (genetics.bwh.harvard.edu/pph2/index.shtml) and SIFT²¹ (sift.jcvi.org) were used (Table 2). Out-of-frame mutations were present in 18/31 (58%) UM: 13 samples had frameshift insertions/deletions, 2 samples splice site mutations, 2 samples nonsense mutations, and 1 sample a read-through mutation (Table 2). The distribution of BAP1 mutations is shown in Supplementary Figure 2. Nineteen out of 31 BAP1 mutations are not yet reported in UM or in any other cancer (Supplementary Table 3). BAP1 promoter methylation analysis was conducted in 3/11 tumors with discrepancy between IHC and sequencing (loss of BAP1 protein expression and no BAP1 mutation), but no hypermethylation was detected (Supplementary Figure 3). SF3B1 mutations were identified at R625 codon in 6/61 (10%) UM: 5/6 SF3B1 mutations occurred in UM with BAP1 wt and positive IHC, and 1/6 in a UM with BAP1 wt but with negative IHC (Figure 1, and Table 2). Five out of 60 (8%) UM had a missense mutation in EIF1AX gene (Figure 1, and Table 2), in either exon 1 (UM32, UM52, UM60) or exon 2 (UM27, UM51). Two out of 5 EIF1AX mutations were found in cases with monosomy 3, BAP1 mutation, and loss of BAP1 nuclear immunostaining.

BAP1 Immunohistochemistry

BAP1 IHC was successful in 62/63 cases (for UM06 no FFPE tumor material was still available), (Figure 1).

Nineteen out of 62 (31%) UM samples showed a BAP1 nuclear positive immunostaining, and 43/62 (69 %) were IHC negative (Figure 2a and 2b, respectively). In positive UM samples, BAP1 staining did not show any intratumoral heterogeneity. Among the 43 UM with negative nuclear IHC, 4 UM samples (UM14, UM23, UM40, UM48) showed a cytoplasmic signal (Table 2, Figure 2c),

and 7 (UM16, UM28, UM31, UM36, UM38, UM43, UM48) with immune-cell infiltration (Table 2, Figure 2d).

Statistical Analysis - Associations between mutations and distant progression free survival (DPFS)

Univariate associations between studied parameters and metastatic disease were performed using the Fisher's exact test (Table 3). Significant associations with DPFS were found for monosomy 3 ($p=0.008$), gain of 8q ($p=0.012$), BAP1 mutation ($p=0.019$), and loss of BAP1 protein expression ($p=0.005$). Conversely, gain of 6p was associated with the absence of liver metastases ($p=0.019$). As regards time-to-event analysis, after a median follow-up time of 3 years (interquartile range, IQR: 2-6 years), median DPFS was 2.5 years (IQR: 1.8–7.7 years). Kaplan-Meier DPFS curves and Hazard Ratios adjusted for age, sex and tumor stage, T (Cox regression) showed that the presence of monosomy 3 was significantly associated to DPFS (HR=6.3, 95%CI: 1.5-27.2), as well as 8q gain (HR=3.6, 95%CI: 1.05-12.6), and BAP1 negative IHC (HR=4.3, 95%CI: 1.4-12.8), while the presence of BAP1 mutation was not statistically significant (HR=1.6, 95%CI: 0.7-3.8) (Figure 3).

DISCUSSION

Specific cytogenetic alterations are associated in UM to metastatic progression²². Also in our UM series, monosomy 3 and gains in 8q were found associated to metastatic UM, and 6p gain to UM with a good prognosis. The relationship between BAP1 loss and UM metastatic progression is reinforced by multiple independent studies^{23,24}. BAP1 protein activity depends on both deubiquitinating domain and nuclear localization. Missense mutations, which are mainly found in UCH domain, can impair the catalytic domain^{25,26}. Frameshift mutations can give rise to abnormal mRNAs, subjected to nonsense-mediated RNA decay²⁷, or resulting in truncated proteins lacking the C-terminal nuclear localization signals or prone to rapid degradation^{28,29}. Altogether, we found 31 BAP1 mutations in UM with monosomy/isodisomy 3. In 6 UM samples, Sanger sequencing identified heterozygous BAP1 mutations. All these samples were characterized by immune-cell infiltration, and in all of them BAP1 IHC clearly showed loss of nuclear immunosignal in UM cells, with infiltrating cells showing positive nuclear immunostaining. In these cases, the most likely explanation for the observed BAP1 mutation heterozygosity could be the presence of wt BAP1 alleles from infiltrating cells rather than BAP1 status heterogeneity in the tumor. Thirteen BAP1 mutations were in-frame mutations, 12 of those clustered in the region spanning exons 4 to 8 of the BAP1 gene, within the UCH domain. In all but one case, the samples with BAP1 missense mutations were IHC negative: UM53, harboring in UCH domain the mutation H94R, predicted damaging by PolyPhen-2 and deleterious by SIFT, showed BAP1 nuclear signal. H94R could likely determine loss of UCH function while maintaining protein expression and nuclear localization. On the other side, all the other 11 in-frame mutations had as consequence the loss of BAP1 protein expression, independently from localization and functionality prediction by Polyphen2 and SIFT. Data about specific BAP1 missense mutations and resulting expression and/or localization of mutated BAP1 protein are not fully in agreement^{16,23,30,31}: BAP1 C91 mutants showed conflicting IHC results, showing positive nuclear signal in some reports^{16,30} and negative in others²³; moreover, BAP1 A95D mutant revealed positive nuclear immunostaining^{16,30} but in another report it was described as structural instable, showing in vitro β -amyloid aggregation and perinuclear accumulation³¹.

Eighteen samples had truncating or read-through BAP1 mutations, and all showed the absence of BAP1 nuclear immunostaining. Previous papers showed that the consequence of out-of-frame mutations is the absence of BAP1 nuclear protein, with some exceptions: Koopmans et al³² reported two hemizygous mutants harboring an out-of-frame deletion in exon 16 with a positive BAP1 staining, and Yavuzyigitoglu et al¹⁶ described two UM cases (both with monosomy 3) expressing nuclear BAP1 protein despite the BAP1 nonsense mutations Q36*, and E406*, respectively. This latter finding is quite surprising, because both Q36* and E406* nonsense mutants are expected to have lost the epitope recognized by the antibody, and, in addition, they should have lost the nuclear localization signals.

Associations between mutations and DPFS are in agreement with previous reports^{8,23,28,33}, with the exception of BAP1 mutation, which did not reach a statistically significant association. Various factors could have influenced the latter result, e.g. heterogeneity in the length of follow-up, the relatively low statistical power which is a common bias of rare disease studies, but, most importantly, in 11/43 (26%) UM with negative BAP1 IHC no BAP1 mutations were found. Among the 11 cases with discrepancy between Sanger sequencing and IHC, 8 had monosomy 3 and 3 partial monosomy 3, 6/11 were metastatic (follow-up 6-39 months), 3/11 were non metastatic (follow-up 11- 23 months), and 2 patients were lost to follow-up. UM cases lacking IHC BAP1 signal without BAP1 mutations were already reported^{16,33,34}, but until now no other mechanisms than mutations are known that could prevent BAP1 expression, i.e. epigenetic changes leading the silencing of the gene, or alterations in non-coding regions that are regulation targets of BAP1 expression.

In our UM series, univariate analysis showed that chromosome 3 monosomy, 8q gain, BAP1 mutations, and loss of BAP1 nuclear immunostaining were all significantly associated with metastatic progression, in agreement to previous studies^{15,32,33-36}. Nevertheless, there is no prognostic factor combination able to identify all metastatic cases: 4/63 (6%) UMs assigned to the good-prognosis group on the basis of their genetic features (disomy 3, BAP1 wt and positive BAP1 IHC) developed liver metastases. Only in one of these cases (UM17), in addition to UM-specific chromosome imbalances, a high instability was observed, with loss of some MLPA-control probes. It is known that rare UM with disomy 3 can develop metastases^{35,37-39}, but there is no evidence on

possibly involved metastatic pathway(s). Although many studies have reported quite large numbers of UM cases, those that have given a detailed mutational and cytogenetic analysis are indisputably fewer⁴⁰. In this view, this study gives a contribution in defining the genetic UM landscape. For prognostication, the presence of monosomy 3 and BAP1 negative IHC are the strongest predictors of metastases, and may have important implications for the implementation of a more intensive patient surveillance and adjuvant therapy. The validity of these observations must be assessed in properly designed clinical trials.

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No conflicts of interest are reported.

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Table 1**Patients, tumor characteristics, and follow-up**

Tumor Id	Age at Diagnosis (Y)	Sex	T	Cell Type	MT	Time To MT (Months)	Follow up (Months)
UM01	64	M	T4A	M	yes	38	38
UM02	62	M	T2A	F	yes	30	30
UM03	69	M	T4	M	yes	24	24
UM04	73	F	T2A	M	yes	93	93
UM05	74	M	T4C	E	yes	47	47
UM06	71	F	-	M	no	0	148
UM07	80	M	T4B	E	yes	29	29
UM08	60	M	T2C	E	yes	97	100
UM09	75	F	T4A	E	yes	26	31
UM10	69	M	T3A	E	no	0	13
UM11	62	M	T4	E	yes	6	6
UM12	78	F	T4A	E	no	0	51
UM13	47	M	T3B	E	yes	25	25
UM14	57	F	T4A	E	yes	14	14
UM15	48	M	T3A	E	no	0	33
UM16	77	M	T4B	E	yes	3	3
UM17	64	M	T3A	E	yes	7	21
UM18	85	M	-	E	no	0	0
UM19	53	M	T2A	E	yes	6	13
UM20	78	M	T3A	E	no	0	3
UM21	74	M	T4	E	yes	27	27
UM22	67	M	T4B	E	yes	17	17
UM23	47	M	T2A	E	no	0	11
UM24	47	M	T3	E	no	0	41
UM25	40	M	T4B	E	no	0	25
UM26	86	M	T4A	E	yes	12	19
UM27	74	M	T3A	M	UN	UN	UN
UM28	49	F	T3A	M	yes	25	25
UM29	41	M	T3A	E	no	0	28
UM30	81	M	-	E	yes	40	43
UM31	78	F	T4A	E	no	0	28
UM32	63	F	T4A	E	no	0	42
UM33	51	F	T3A	E	yes	0	0
UM34	74	F	T2A	M	yes	64	64
UM35	28	F	-	M	UN	UN	UN
UM36	66	M	T3A	M	no	0	22
UM37	79	M	T3A	M	yes	17	20
UM38	71	M	-	E	yes	25	39
UM39	66	F	T3A	E	no	0	21
UM40	66	F	T4	E	yes	6	19
UM41	59	M	T4A	E	no	25	25
UM42	83	F	T3A	E	no	0	36

Table1. (Continued)

Tumor Id	Age at Diagnosis (Y)	Sex	T	Cell Type	MT	Time To MT (Months)	Follow up (Months)
UM43	30	M	T3A	E	no	0	23
UM44	75	F	-	M	yes	11	11
UM45	28	F	T3A	E	yes	13	28
UM46	89	F	T4B	E	no	0	23
UM47	47	M	T2	E	no	0	52
UM48	58	M	T4A	E	yes	7	7
UM49	41	M	T3A	E	yes	26	39
UM50	64	F	T4A	E	yes	6	23
UM51	59	M	T3A	M	no	0	8
UM52	35	F	T3A	E	no	0	1
UM53	64	F	T4A	M	no	0	22
UM54	71	F	T4	E	yes	22	22
UM55	28	M	T4A	E	no	0	22
UM56	68	F	T4A	E	no	0	43
UM57	64	M	T4A	E	no	0	35
UM58	78	M	T4A	E	yes	0	0
UM59	40	F	T4A	E	yes	26	38
UM60	52	M	T3A	E	yes	0	36
UM61	66	M	T2A	E	no	0	73
UM62	51	M	-	E	yes	23	27
UM63	84	M	T4B	E	yes	30	34

M, male; F, female; T, tumor stage; E, epithelioid cell type; S, spindle cell type; M, mixed epithelioid and spindle cells; MT, metastases; UN, unknown.

Table 2

Genetic Analysis of 63 UM studied

Tumor ID	MTS	Chrom. 3 Status	GNAQ protein	GNA11 protein	EIF1AX protein	SF3B1 protein	BAP1 sequencing	BAP1 protein	Polyphen2/ SIFT prediction	BAP1 nuclear IHC	Immune-Infiltr.
UM01	yes	M3	wt	Q209L	wt	wt	wt	wt		pos	
UM02	yes	ISO 3	wt	Q209L	wt	R625C	wt	wt		neg	
UM03	yes	M3	wt	Q209L	wt	wt	c.1817_1821del	A606Gfs*35		neg	
UM04	yes	M3	wt	wt	wt	wt	c.265_300del	N89_L100del		neg	
UM05	yes	M3	wt	wt	wt	wt	c.290T>C	L97P	Probably damaging/ Damaging	neg	
UM06	loss FU	D3	wt	wt	wt	wt	wt	wt (K139K)		-	
UM07	yes	M3	wt	Q209L	wt	wt	c.2089T>C	S697P	Probably damaging/ Tolerated	neg	
UM08	yes	M3	wt	wt	wt	wt	c.287_307del	L96_N102del		neg	
UM09	yes	M3	wt	Q209L	nd	nd	nd	nd		neg	
UM10	loss FU	M3	wt	wt	wt	wt	wt	wt		neg	
UM11	yes	M3	wt	wt	wt	wt	c.638G>C	R213P	Probably damaging/ Damaging	neg	
UM12	no	D3	wt	wt	wt	R625C	wt	wt		pos	
UM13	yes	D3	Q209P	wt	wt	wt	wt	wt		pos	
UM14	yes	M3	wt	wt	wt	wt	nd	nd		neg ^b	
UM15	no	D3	wt	Q209L	wt	R625H	wt	wt		pos	
UM16	yes	M3	Q209L	wt	wt	wt	c.425del ^a	N142Ifs*45		neg	+
UM17	yes	D3	wt	wt	wt	wt	wt	wt		pos	
UM18	loss FU	M3	wt	wt	wt	wt	wt	wt		neg	
UM19	yes	M3	Q209P	wt	wt	wt	wt	wt		neg	
UM20	no	ISO 3	Q209P	wt	wt	wt	c.1074-1081del	A359Rfs*36		neg	
UM21	yes	M3	wt	Q209L	wt	wt	c.188C>G	S63C	Probably damaging/ Tolerated	neg	
UM22	yes	M3	wt	Q209L	wt	wt	c.233A>G	N78S	Possibly damaging/ Tolerated	neg	
UM23	no	M3	wt	wt	wt	wt	wt	wt		neg ^b	
UM24	no	D3	Q209P	wt	wt	R625H	wt	wt		pos	
UM25	no	D3	Q209L	wt	wt	nd	wt	wt		pos	
UM26	yes	M3	wt	Q209L	wt	wt	c.175_179del	R59Kfs*8		neg	
UM27	no	D3	wt	wt	G15N	wt	wt	wt		pos	
UM28	yes	M3	wt	Q209L	wt	wt	c.466C>T ^a	Q156*		neg	+

Table 2. (Continued)

Tumor ID	MTS	Chr 3 Status	GNAQ protein	GNA11 protein	EIF1AX protein	SF3B1 protein	BAP1 sequencing	BAP1 protein	Polyphen2/ SIFT prediction	BAP1 nuclear IHC	Immune-Infiltr.
UM29	no	M3	wt	R183C	nd	wt	c.335T>G	L112R	Probably damaging/ Damaging	neg	
UM30	yes	M3	Q209P	wt	wt	wt	c.356_358del	T119del		neg	
UM31	no	M3	wt	Q209L	wt	wt	c.145del ^a	L49Cfs*23		neg	+
UM32	no	M3	Q209P	wt	G6R	wt	c.79del	V27Cfs*45		neg	
UM33	yes	ISO 3	Q209L	wt	wt	wt	c.145del	L49Cfs*23		neg	
UM34	yes	ISO 3	Q209P	wt	wt	wt	c.1499_1515del	G500Afs*31		neg	
UM35	no	M3	wt	wt	wt	wt	wt	wt		neg	
UM36	no	M3	wt	Q209L	wt	wt	c.629T>A ^a c.627C>A ^a	I210N (V209V)	Probably damaging/ Damaging	neg	+
UM37	yes	M3	Q209L	wt	wt	wt	c.283G>C c.1356C>T	A95P (L452L)	Probably damaging/ Damaging	neg	
UM38	yes	pM3	Q209P	wt	wt	wt	c.681C>T c.2163T>C	wt (R227R) (S721S)		neg	+
UM39	no	M3	wt	wt	wt	wt	wt	wt		pos	
UM40	yes	pM3	wt	Q209L	wt	wt	wt	wt		neg ^b	
UM41	yes	M3	wt	Q209L	wt	wt	c.327_328insAG	P110Sfs*4		neg	
UM42	no	pM3	R183Q	wt	wt	R625H	wt	wt		pos	
UM43	no	M3	wt	wt	wt	wt	c.438-2A>G ^a	SA		neg	+
UM44	yes	M3	Q209P	wt	wt	wt	c.2189G>T	*730Lext*205		neg	
UM45	yes	M3	Q209P	wt	wt	wt	c.760_763del	T254Yfs*2		neg	
UM46	no	M3	Q209P	wt	wt	wt	wt	wt		neg	
UM47	no	D3	wt	Q209L	wt	wt	wt	wt		pos	
UM48	yes	M3	wt	wt	wt	wt	c.976del ^a	A323Pfs*12		neg ^b	+
UM49	yes	M3	wt	R183C	wt	wt	nd	nd		pos	
UM50	yes	M3	wt	Q209L	wt	wt	wt	wt		neg	
UM51	no	M3	Q209P	wt	G9N	wt	c.681_697del	F228Gfs*9		neg	
UM52	no	D3	wt	Q209L	G6V	wt	wt	wt		pos	
UM53	no	M3	Q209P	wt	wt	wt	c.281A>G	H94R	Probably damaging/ Damaging	pos	
UM54	yes	pM3	wt	wt	wt	wt	wt	wt		neg	
UM55	no	M3	wt	Q209L	wt	wt	c.375+1G>A	SA		neg	
UM56	no	D3	Q209L	wt	wt	wt	wt	wt		pos	
UM57	no	D3	wt	Q209L	wt	R625C	wt	wt		pos	
UM58	yes	M3	wt	Q209L	wt	wt	c.503del	F168Sfs*19		neg	
UM59	yes	M3	wt	Q209L	wt	wt	c.7A>T	K3*		neg	

Table 2. (Continued)

Tumor ID	MTS	Chrom. 3 Status	GNAQ protein	GNA11 protein	EIF1AX protein	SF3B1 protein	BAP1 sequencing	BAP1 protein	Polyphen2/SIFT prediction	BAP1 nuclear IHC	Immune-Infiltr.
UM60	yes	D3	wt	Q209L	K3E	wt	wt	wt		pos	
UM61	no	D3	wt	Q209L	wt	wt	wt	wt		pos	
UM62	yes	D3	wt	wt	nd	wt	wt	wt		pos	
UM63	yes	M3	Q209L	wt	wt	wt	c.580G>A	G194R	Probably damaging/Damaging	neg	

FU, follow up; D, disomy; M, monosomy; pM, partial monosomy; FS, frameshift; IF, in frame; NS, nonsense; RT, read-throughs; syn, synonymous; E, exon; I, intron; nd, not done. Numbering of mutations on the genomic level refers to build GRCh37/hg19 (ensemble/UCSC); ^a, heterozygous mutation; ^b, BAP1 IHC cytoplasmic signal

Table 3 Association of clinical and molecular characteristics with metastatic disease in univariate analyses (Fisher's exact test)

	No MTs	MTs		two-tailed P value
Sex				
M	17	23	40	P=0.79
F	11	12	23	
Position				
choroid+ciliar body	1	4	5	P=0.36
choroid	28	30	58	
Extrinscation				
no	26	21	47	P=0.28
yes	3	6	9	
Cell type				
epithelioid	23	26	49	P=0.55
mixed epithelioid/fused	8	6	14	
GNAQ				
wt	19	24	43	P>.99
mut	9	11	20	
GNA11				
wt	18	20	38	P=0.61
mut	10	15	25	
BAP1 (Sanger seq)				
wt	19	10	29	P=0.019
mut	10	21	31	
BAP1 IHC				
pos	14	5	19	P=0.005
neg	14	29	43	
SF3B1				
wt	22	33	55	P=0.08
mut	5	1	6	
EIF1AX				
wt	23	32	54	P=0.16
mut	4	1	6	
Chr3 status				
D	12	4	16	P=0.008
M	16	30	46	
1p				
wt	22	25	48	P=.76
1p-	7	6	12	
6p				
wt	17	27	46	P=0.019
6p+	12	4	14	
8q				
wt	14	5	19	P=0.012
8q+	15	27	41	

MT, metastasis; M, male; F, female; D, disomy; M, monosomy.

Figure legends

Figure 1. Overview of chromosome imbalances, gene mutations, and metastatic progression

Squares in full grey color indicate the presence of the specific chromosome imbalances; squares with different graphic patterns indicate the presence of a mutation; black square, metastatic UM cases; squares with different graphic patterns indicate in BAP1 IHC row indicate BAP1 negative nuclear IHC signal; ND, not determined; ISO, isodisomy; P, partial gain/loss.

Figure 2. BAP1 IHC in UM FFPE sections.

a) BAP1 positive nuclear IHC signal in UM13, characterized by disomy 3 and BAP1 wt. b) Lack of nuclear BAP1 IHC signal in UM32, with monosomy 3 and BAP1 c.79del mutation. c) Cytoplasmic BAP1 IHC signal in UM40, characterized by a partial monosomy 3 and BAP1 wt. d) IHC staining of UM16, a case with monosomy 3 and BAP1 c.425del mutation: tumor cells are BAP1 IHC negative, and infiltrating cells show BAP1 positive nuclear IHC signal (left panel); the electropherogram shows a heterozygous deletion (right panel). In all the cases with negative BAP1 IHC, BAP1 nuclear IHC signal in endothelial cells represents the internal positive control.

Figure 3. Kaplan-Meier DPFS curves and Hazard Ratios

Kaplan-Meier Distant Progression Free Survival (DPFS) curves and Hazard Ratios adjusted for age, sex and tumor stage (T) (Cox regression) (HR*) for: a) chromosome 3 status (monosomy/disomy); b) 8q status (disomic 8q/8q gain); c) BAP1 IHC nuclear signal (positive/negative); d) BAP1 mutation by Sanger sequencing (wt/mutated).